Novel ssDNA Viruses Detected in the Virome of Bleached, Habitat-Forming Kelp *Ecklonia radiata*

Douglas T. Beattie [1]*, Tim Lachnit [1,2], Elizabeth A. Dinsdale [3], Torsten Thomas [1] and Peter D. Steinberg [1,4]

[1] Centre for Marine Bio-Innovation and School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW, Australia, [2] Zoological Institute, Christian-Albrechts-University Kiel, Kiel, Germany, [3] Department of Biology, San Diego State University, San Diego, CA, United States, [4] Sydney Institute of Marine Science, Mosman, NSW, Australia

Kelp forests provide essential habitats for organisms in temperate rocky shores. Loss of kelp forests has occurred over large areas in a number of temperate regions, including in Australia, where the dominant kelp *Ecklonia radiata* has been lost from substantial areas of the shoreline. Loss of *E. radiata* has been associated with environmental stressors, including increased temperature and anthropogenic contaminants, as well as biological factors, such as herbivory. Disease may also play a role, but there is little information on the role of disease in the loss of kelp from coastal ecosystems or on the potential role of pathogenic microorganisms, such as viruses. *E. radiata* across much of its distribution in Australia can develop a "bleached" phenotype, which may be a disease. To investigate whether the phenotype was associated with a potential viral agent, we shotgun sequenced viral particles that were isolated from kelp with normal (healthy) and bleached phenotypes. Each virome consisted of ∼380,000 reads, of which ∼25% were similar to known viruses. All samples were dominated by bacteriophages, but novel ssDNA virus sequences were detected that were almost exclusively in viromes from the bleached kelp phenotype. These ssDNA viruses are covered by 11 contigs that contained complete capsids and characteristic *rep* genes that were 30–60% similar to those of circular, Rep-encoding ssDNA viruses (CRESS-DNA viruses). CRESS-DNA viruses have not previously been described from macroalgae, and the *rep* genes were similar to CRESS-DNA viruses from marine water samples, snails, crabs, anemones, but also dragonflies. This raises the interesting possibility that the kelp could be a vector of the CRESS-DNA viruses to other organisms that are associated with the bleached state.

Keywords: circovirus, microbial ecology, virome, kelp, marine disease, metagenome, CRESS-DNA, viral ecology

INTRODUCTION

Ecklonia radiata is a large, stipitate brown alga (class *Phaeophyceae*, order *Laminariales*, or commonly, kelp), and the dominant primary habitat-forming organism over 71,000 km^2 of the temperate rocky coastlines of Australia (Bennett et al., 2016). Kelps, such as *E. radiata,* provide habitat and nutrients that are essential to coastal food webs (Steneck et al., 2002), and the importance of *E. radiata* habitats in temperate Australia has led to them being described as

"The Great Southern Reef" (Bennett et al., 2016). Fisheries, tourism, and other industries taking place within these habitats are estimated to be worth AU$10 billion annually to the Australian economy (Bennett et al., 2016).

In recent years there has been a loss of kelp cover in temperate regions of Australia from locations across much of the range of *E. radiata*, from Perth on the West coast, along the Southern coast, up to the Solitary Islands on the East coast (Connell et al., 2008; Ling and Johnson, 2012; Vergés et al., 2016; Wernberg et al., 2016). Loss of kelp results in major losses in biodiversity, ecosystem function, and associated fisheries (Andrew and O'Neill, 2000; Bishop et al., 2010; Burge et al., 2014; Vergés et al., 2016; Wernberg et al., 2016), and in Australia loss of *E. radiata* has been associated with a number of environmental stressors. This includes stressors associated with urbanization, such as increased nutrients and sedimentation of the coastal region (Connell et al., 2008), and ocean warming, through both direct physiological effects of temperature as well as indirect effects through range extensions of herbivorous sea urchins or fishes, which consume large areas of kelp forests (Wernberg et al., 2011; Ling and Johnson, 2012; Vergés et al., 2016). Though relatively little studied in seaweed systems, microbial disease may also play a role in loss of habitat-forming seaweeds (Egan et al., 2014). Disease in marine systems appears to be increasing globally, including in communities that are dominated by primary habitat-forming organisms, such as coral reefs and kelp forests (Rosenberg et al., 2007; Vega Thurber et al., 2009; Krediet et al., 2013), and disease in seaweeds has been linked to anthropogenic stressors, such as ocean warming (Campbell et al., 2011; Case et al., 2011).

Microbial communities are integral to the health of all organisms, such that hosts and their associated microbial communities (microbiome) are increasingly being treated as a holistic system, or holobiont (Egan et al., 2013). This approach is now common for understanding the interaction between marine habitat-forming organisms, such as corals, seaweeds, or sponges (Bourne et al., 2009; Egan et al., 2013; Webster and Thomas, 2016). Disturbances to the microbiome of these organisms are associated with stress and disease of the host (Morris et al., 2016). For example, disruption of the microbiome of the Australian macroalga *Delisea pulchra* leads to bleaching and reduced reproductive capacity (Egan et al., 2013; Campbell et al., 2014; Wahl et al., 2015), and microbial pathogens cause bleaching of *D. pulchra* in both the field (Campbell et al., 2011) and in laboratory inoculation experiments (Kumar et al., 2016). Disruption of the holobiont and resultant disease is increasingly seen as critical for other marine habitat forming organisms.

We have recently identified a bleaching phenotype in *E. radiata*, which occurs widely across its biogeographic range and which is characterized by bleached areas of the thallus with reduced photosynthetic capacity (Marzinelli et al., 2015). The bacterial components of the microbiome of bleached vs. non-bleached *E. radiata* were distinct, suggesting that the microbiome of the algae undergoes a shift associated with the disruption of normal function (e.g., photosynthesis; Marzinelli et al., 2015). However, specific bacterial pathogens, which induce bleaching, have not yet been extensively characterized. Other components of

the microbiome may also affect the health of hosts. In particular, viruses are likely ubiquitous components of marine holobionts and are now emerging as pathogens or potential pathogens in a diverse range of marine organisms, including archaea (Rohwer and Thurber, 2009), microalgae (Brussaard, 2004; Weynberg et al., 2017), sea stars (Hewson et al., 2014), and macroalgae (Lachnit et al., 2015).

Viruses consist of two major groups, bacteriophages that infect bacteria and viruses that infect eukaryotes. The tailed bacteriophages with dsDNA genomes, *Caudovirales*, include the families *Siphoviridae*, *Myoviridae*, and *Podoviridae*. Bacteriophages also include *Inoviridae*, which possess ssDNA genomes, and together these bacteriophages form a major component of marine viral assemblages (Mann, 2005; Dinsdale et al., 2008b; Roux et al., 2012; Xue et al., 2012). Abundant eukaryote viruses detected in algae are from the family *Phycodnaviridae*, which have a dsDNA genome of 100–550 kb and infect both unicellular and multicellular algae (Wilson et al., 2009). *Phycodnaviridae* have been investigated in the filamentous brown-alga *Ectocarpus siliculosus*, a model organism, and a few genomes have been sequenced (Van Etten et al., 1991; Müller and Frenzer, 1993; Easton et al., 1997; Kapp et al., 1997; Maier et al., 1998; Delaroque and Boland, 2008). However, few studies have been conducted on macroalgae from the field (e.g., viruses in *Laminaria digitata* McKeown et al., 2017) and none on *E. radiata*.

ssDNA viruses, which have small circular genomes (1–5 kb), are an emerging group of viruses that are implicated in marine disease and mass mortalities across a number of marine phyla, including fishes, molluscs, echinoderms, and crustaceans (Hasson et al., 1995; Friedman et al., 2005; McLoughlin and Graham, 2007; Lang et al., 2009). ssDNA viruses include a subgroup which possess circular genomes, and a particular replication initiation gene, *rep*. These are therefore called Circular Rep-Encoding ssDNA (CRESS-DNA) viruses (Rosario et al., 2009; Delwart and Li, 2012; Labonté and Suttle, 2013; Simmonds et al., 2017). These include the *Geminiviridae, Nanoviridae, Circoviridae,* and *Genomoviridae*, which employ rolling-circle replication (RCR), facilitated by the Rep protein (Rosario et al., 2012b). In addition to the Rep protein, CRESS-DNA viruses have a characteristic stem-and-loop secondary structure, which facilitates RCR. The loop region contains a conserved nonanucleotide motif, NANTATTAC (Labonté and Suttle, 2013). The *Circoviridae* and *Nanoviridae* are diverse in aquatic and marine environments, including ocean, freshwater, wastewater, deep-sea vents, Antarctic lakes and ponds, and hot springs (Angly et al., 2006; Rosario et al., 2009, 2012a; Cassman et al., 2012; Liu et al., 2012; Labonté and Suttle, 2013).

Here we investigate the viruses associated with bleached and non-bleached *E. radiata* from Sydney, Australia. First, virus particles were extracted from kelp tissue. DNA was extracted from these and amplified using whole-genome amplification (WGA), which is biased toward small and circular genomes (Sabina and Leamon, 2015), but thus aids in detection of potentially rare and small genomes in metagenomic samples (Kim et al., 2008; Cheval et al., 2011; Rosario et al., 2012a; Fahsbender et al., 2015). This was followed by shotgun

sequencing and metagenomic analysis. We describe the general features of the kelp virome, and test whether components of the virome differ between the non-bleached and bleached phenotype, in order to explore whether viral pathogens may be a possible cause of the bleaching phenotype. Our results show that circoviruses were nearly absent in the non-bleached kelp, but in high abundance in the bleached kelp, and we describe these novel viruses in detail.

METHODS

Sampling

Individual thalli of *E. radiata* were sampled from Long Bay in Sydney, Australia (−33.97, 151.26) during June 2014 at a depth of 3 m. The water temperature at the time of sampling was 16°C. Tissue samples of blades were taken from three bleached individuals and three non-bleached, healthy, thalli. These phentoypes are described in Marzinelli et al. (2015). Viral particles were isolated from kelp tissue as described in Lachnit et al. (2015).

DNA Extraction and Metagenomic Sequencing

Viral DNA was extracted from virus particles in SDS-extraction buffer (1% SDS, 200 mM Tris, 50 mM EDTA, pH 7.5) in the presence of 1% (w/v) polyvinylpyrrolidone (MW 40,000), 1% 2-mercaptoethanol and proteinase K (0.5 mg ml^{-1}) at 37°C for 30 min, followed by 56°C for 15 min. Double the volume of DNA-extraction buffer (2.6 M NaCl, 150 mM Tris, 75 mM EDTA, 2.5% cetyltrimethylammonium bromide (CTAB), 1% 2-mercaptoethanol, pH 7.5) was added and samples were incubated for another 15 min at 56°C. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the solution, mixed and centrifuged at 12,000 RCF at room temperature for 5 min. The chloroform:isoamyl alcohol extraction of the supernatant was repeated three times to remove contaminating polysaccharides and proteins. The supernatant was transferred into a new tube and the nucleic acids were precipitated by the addition of 0.7 volume of isopropanol. After an incubation step at 20°C for 2 h nucleic acids were pelleted by centrifugation at 14,000 RCF at 4°C for 20 min. The pellet was washed with 75% (v/v) ethanol, air dried and dissolved in 30 μl molecular grade water.

All samples underwent whole genome amplification steps using a REPLI-g Mini Kit (QIAGEN), where the reaction was conducted using 20 μL of denatured DNA, incubating the reaction mix at 30°C for 16 h. DNA was frozen and submitted to the Ramaciotti Centre at UNSW for library preparation using a TruSeq Low Input kit (Illumina). Shotgun metagenomic sequencing was conducted using an Illumina MiSeq sequencer with 2 × 250 bp chemistry. A total of six viral metagenomes (or viromes) were sequenced, three from kelp with the bleached phenotype and three from the non-bleached phenotype. The reads were processed using Prinseq (Schmieder and Edwards, 2011) by trimming the ends where the quality score was below 20, and removing exact duplicates. The paired-end reads

were assessed using default settings on PEAR (Zhang et al., 2014) to determine if they could be joined to form longer sequences.

Virome Profile Analysis

Several bioinformatics approaches were used to identify the types of phage and eukaryotic viruses in the kelp viromes. First, the reads were aligned against the NCBI non-redundant protein database (NR), downloaded on May 05 2017. Alignment was performed using BLASTx (Altschul et al., 1990) with an e-value of 10^{-5}. Alignment matches were screened to remove all non-viral similarities found. Alignment results were analyzed using MEGAN (Huson et al., 2016) to infer best taxonomic and/or functional assignments. The Least Common Ancestor (LCA) parameters was used with a minimum score of 50, max expected e-value 10^{-5}, top 10%, LCA 100%, minimum complexity 0. To detect significant differences in the relative abundances of assignments to each virus family, STAMP (Parks et al., 2014) was used to perform two tailed Welch's *t*-test ($\alpha = 0.05$) with Bonferroni correction to compare the bleached and non-bleached kelp viromes.

To assess possible contamination of the viromes by host genomic or bacterial DNA, the entire set of similarities found, including similarities to viruses and non-viruses, was considered. Sequences, which had higher alignment bitscores to non-viruses, were aligned against all the brown-alga sequences in NCBI (downloaded on May 05 2017), using BLASTn with an e-value of 10^{-5}. The sequences with <95% similarity to *Ecklonia spp.* suggested that they are not part of the brown algae. This threshold was chosen as essential genes (e.g., *rbcL*) of different species of *Eckonia*, and even some distally related members of the brown algae are >95% similar (Rothman et al., 2015). Decontamination programs (e.g., DeconSeq Schmieder and Edwards, 2011) were not employed to automate the removal of potential bacterial contamination, as genes are often shared between bacteriophage and their hosts. Instead, more information on the origin of the reads was obtained by assembly and assessment of the genomic content (see below), and contamination was retrospectively removed from the results and analyses.

The individual metagenomes were assembled to provide longer contigs using SPAdes 3.9.0 (using default parameters; Bankevich et al., 2012). SPAdes was chosen as it performs better than other assemblers on virome data (Hesse et al., 2017; Papudeshi et al., 2017; Vollmers et al., 2017). Reads were mapped to the contigs using Bowtie 2.2.5 (Langmead and Salzberg, 2012) and visualized using Tablet (Milne et al., 2009) to assess the evenness of read coverage and to identify possible chimeric assemblies, which were removed from further analysis. To investigate the distribution of viruses between the bleached and non-bleached kelp, the contigs were aligned to the NR database as described above. The contigs and their component reads were considered contamination and removed from the analysis and results if (a) the alignment bitscore to a virus reference was less than to a non-virus sequence, and (b) the alignment matches did not contain core

genes of the assigned virus taxon e.g., phage-tail genes of *Caudovirales*.

Viral Genome Reconstruction

All six metagenomes were pooled and cross-assembled with SPAdes 3.9.0 to obtain longer contigs and/or complete genomes. Assessment and alignments of the cross-assembled contigs were performed as described above. At this stage of the analysis, circoviruses were identified as the only part of the eukaryotic DNA virus community that varied in abundance between bleached and non-bleached kelp, and therefore were investigated in more detail. Open reading frames (ORFs) on the circoviral contigs were detected using Prodigal (Hyatt et al., 2010) with a length of > 50 amino acids. Predicted protein sequences were aligned against one another, and their reference sequences using the MUSCLE aligner with default settings (Edgar, 2004). To visualize the diversity of the ORFs a phylogenetic tree was produced using the Jukes-Cantor genetic distance model and the neighbor-joining algorithm with 100 bootstraps. *Circoviridae* contigs were submitted to Mfold (Zuker, 2003) to determine their stem-and-loop secondary structure. The loop motif was compared across the *Circoviridae* group through alignment using MUSCLE.

RESULTS

Overall Composition of the Virome of *Ecklonia radiata*

The six *E. radiata* viromes contained an average of 386,000 reads with an average length of $149 \pm 2SD$ bp. (Supplementary information S1). No reads were removed through quality control, although 14% of the reads were trimmed up to 10 bp where the read quality was <20. Most paired reads could not be joined, and so forward-reads were only used in alignment searches. Analysis of alignment results found $81,256 \pm 22,106$ virus-like reads per metagenome (~20% of the sequences were similar to the NR database). The majority (86–94%) of virus-like reads were assigned to bacteriophage in the order *Caudovirales* (**Figure 1**). In order of abundance, similarities were detected to the viral groups *Siphoviridae* (average of 48% of virus-assigned reads), *Myoviridae* (16%), unclassified bacteriophages (12%), *Inoviridae* (8%), *Podoviridae* (7%), followed by *Circoviridae* (7%). Within the *Caudovirales*, reads similar to *Siphoviridae* were more abundant (adjusted *p-value* = 0.003) in the non-bleached kelp phenotype, however the total number of bacteriophage reads within the *Caudovirales* did not significantly differ between non-bleached and bleached kelp viromes. In the eukaryotic viral fraction, reads similar to *Phycodnaviridae* and other large dsDNA viruses were lower in abundance (<2% of reads assigned) compared to the bacteriophages and did not differ in abundance between viromes of non-bleached and bleached kelp (adjusted *p-value* = 0.785). Sequences of ssDNA viruses belonging to the *Circoviridae* were rare overall (0.48% of total reads), but were almost exclusively in bleached kelp viromes. Six reads were assigned to *Circoviridae* from a single non-bleached kelp

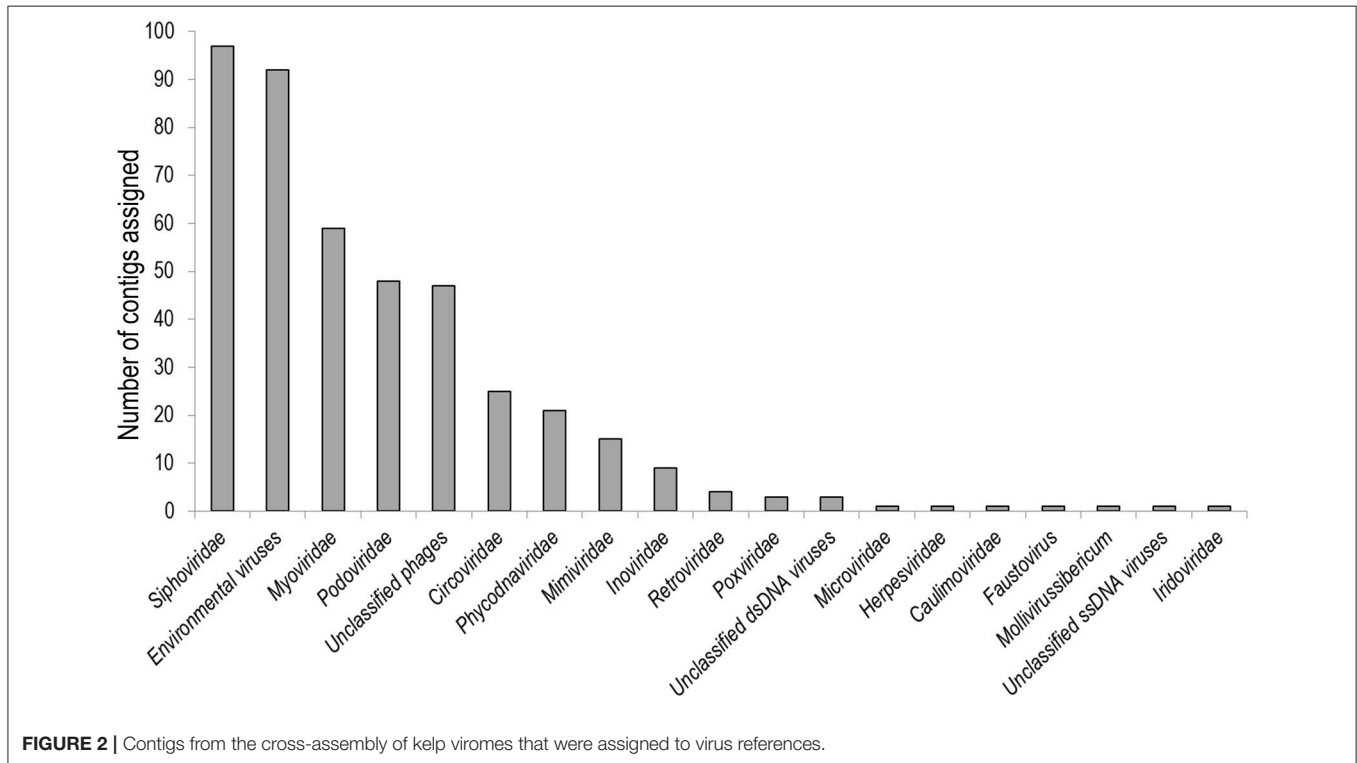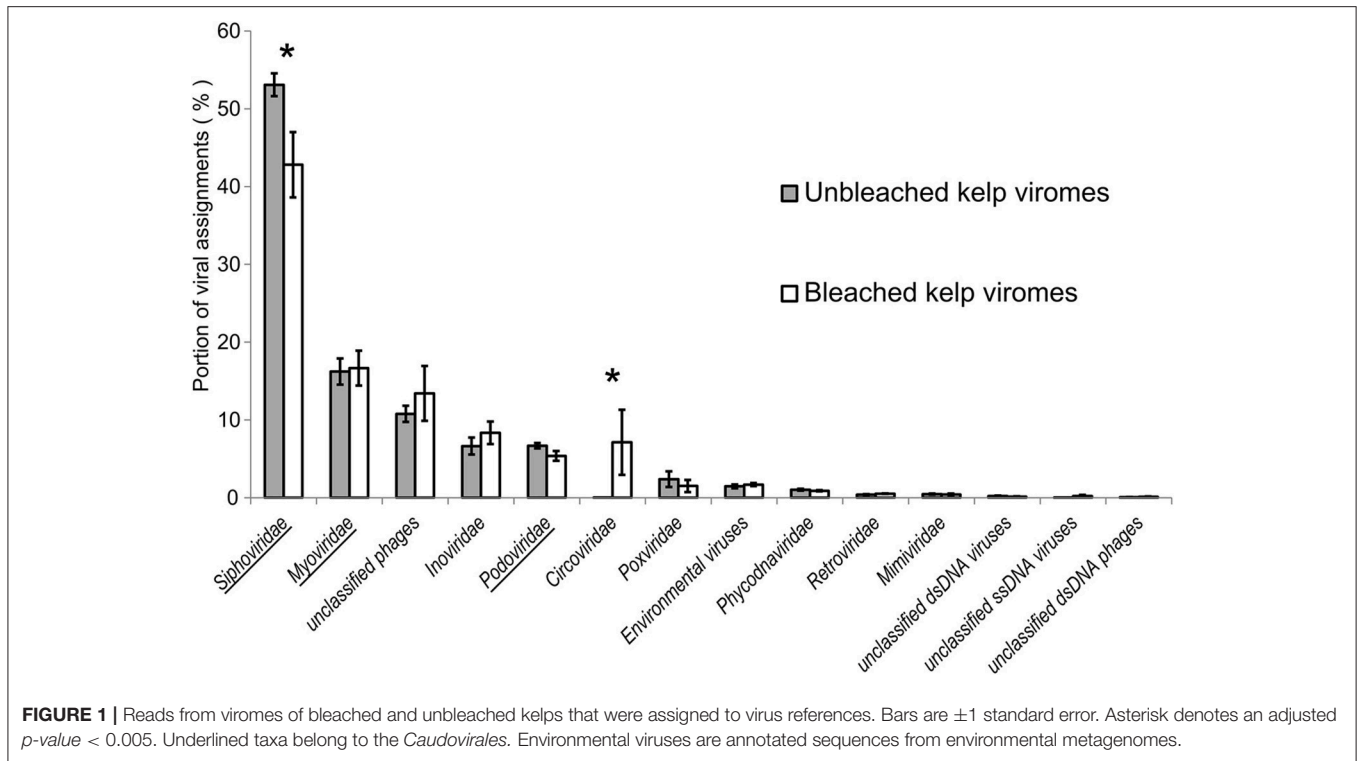virome, whereas 6,403 reads were assigned from bleached kelp viromes.

Assembly of all reads per individual metagenome provided an average of $4,011 \pm 351$ contigs of $38,709 \pm 6,392$ bp length. The taxonomic assignment of the contigs mirrored that of the reads (Supplementary information S2). Bacteriophage possessed the greatest amount of read coverage in the contigs (60%), followed by the *Phycodnaviridae* (8%) and *Circoviridae* (6%). The *Circoviridae* contigs ($n = 42$) were assembled only in the bleached kelp viromes, with the exception of a single contig from one non-bleached kelp virome. Similar to the read data, *Circoviridae* contigs were the only eukaryotic viral group that varied in coverage between the bleached and non-bleached kelp phenotypes (adjusted *p-value* = 0.002).

Cross-assembly of all six metagenomes yielded 7,561 contigs, the longest being 246,893 bp (N_{50} 20,791 bp, N_{95} 246 bp). When aligned against an NCBI virus-only protein database, 847 contigs showed similarity to viruses (**Figure 2**). In descending order of abundance these were assigned to *Siphoviridae* (97 contigs), environmental viruses from metagenomes (92), *Myoviridae* (59), *Podoviridae* (48), unclassified bacteriophages (47), *Circoviridae* (26), *Phycodnaviridae* (21), *Mimiviridae* (15), *Inoviridae* (9) and other virus families with very low abundances (total of 434).

We assessed contamination by regarding the contig similarities to virus and non-viruses. Two thousand two hundred and sixty-seven contigs of the total 7,561 were given a taxonomic assignment through alignment to NR (Supplementary information S3). Eight hundred and forty-seven contigs had similarity to viruses. Of these, 146 contigs had no similarity to other references, 576 contigs were also similar to bacteria and 125 contigs were also similar to eukaryotes. The majority (36%) of contigs similar to both viruses and eukaryotes were similar to *E. siliculosus* Virus-1. Four contigs had both higher alignment bitscores to brown-alga sequences, and did not include characteristic virus genes or conserved domains and were thus assumed to be eukaryotic. The contigs with similarity to both viruses and bacteria (576) were examined further, finding 113 contigs with higher bitscores to bacterial references and no characteristic bacteriophage genes, and assumed to be from bacterial cells. Contigs and their constituent reads that were considered eukaryotic or bacterial contamination were removed from the analyses and results of virus abundances and gene content. Contigs that were assigned as circoviruses had no other similarities to references in NR, and as the circoviruses were the only eukaryotic virus that showed variation between kelp phenotypes, these were analysed in more detail.

Investigation of *Circoviridae*-Like Contigs

The *Circoviridae* contigs ($n = 26$) were 739–5,420 bp long, and contained up to two ORFs. The first ORF was 855 bp, and the only translated similarity between these and reference proteins were two ORFs with similarity to putative CRESS-DNA virus capsid genes from environmental metagenomes. These showed 30% pairwise identity to putative capsid genes of a circular virus associated with a sea anemone (Genbank accession: YP_009163900.1) and a circular ssDNA virus isolated from an Antarctic ice shelf pond (Genbank accession: YP_009047127.1).

FIGURE 1 | Reads from viromes of bleached and unbleached kelps that were assigned to virus references. Bars are ±1 standard error. Asterisk denotes an adjusted *p*-value < 0.005. Underlined taxa belong to the *Caudovirales.* Environmental viruses are annotated sequences from environmental metagenomes.

FIGURE 2 | Contigs from the cross-assembly of kelp viromes that were assigned to virus references.
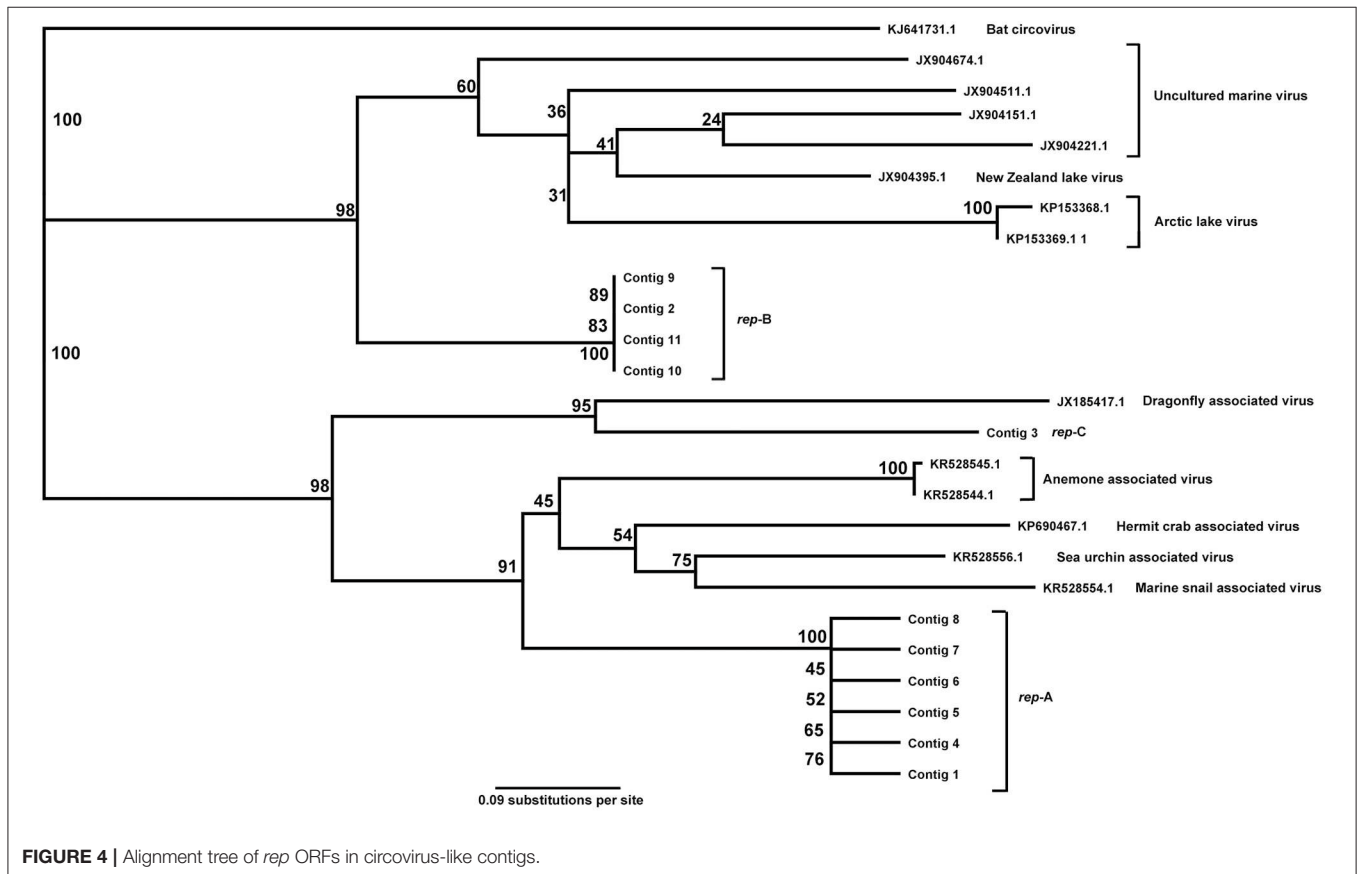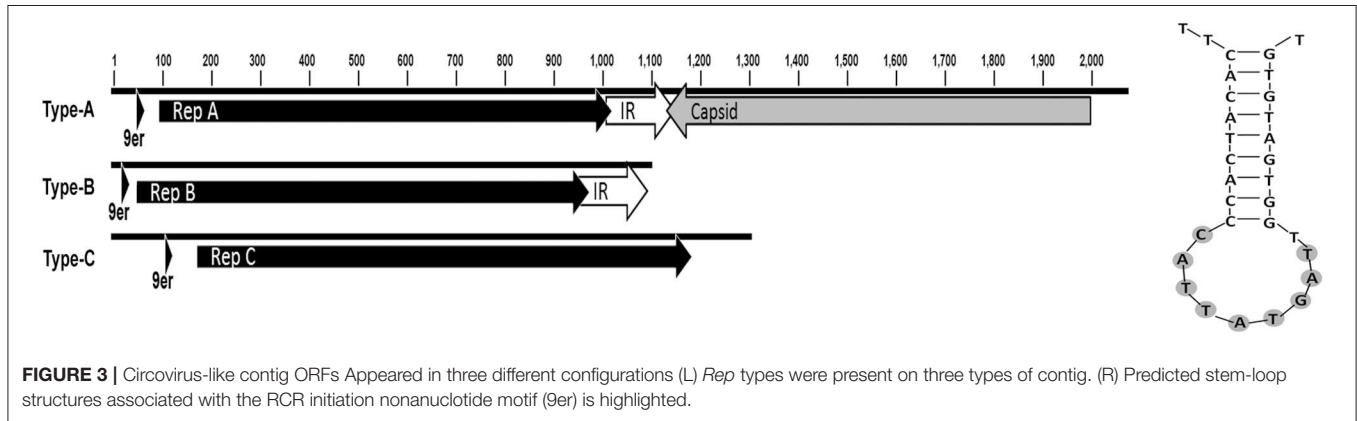
The second ORF was 305–336 amino acids and was the characteristic *rep* genes of CRESS-DNA viruses. The *rep* genes were found in three different lengths (915 bp, 924 bp, and 1,008 bp) suggesting three genotypes, which we refer to from hereon as A-C. There were three contig configurations of the circovirus sequences: Type-A (8 contigs), Type-B (2 contigs)

and Type-C (single contig; **Figure 3**). Type-A contained both the capsid and *repA*, in reverse orientation with a conserved intergenic region (IR) between the two ORFs. The IR of the eight contigs shared a pairwise identity of 70%. Type-B contained *repB* and the IR. Type-C contained only *repC* and no capsid sequences or the IR. The origin of replication (*ori*), included the characteristic nonanucleotide stem and loop structures involved in rolling circle replication of CRESS-DNA viruses and was located upstream of the *rep* gene in all contigs (**Figure 3**). The nonanucleotide motif in each contig was TAGTATTAC. The other 15 of the 26 circovirus-like contigs that did not contain

complete ORFs were found to contain fragments of *rep* and capsid genes of circoviruses, suggesting incomplete assembly.

The *rep* genes from the 11 contigs and the closest reference sequences in the database were aligned. The *rep* genes formed three clusters with each cluster showing >95% similarity. Between all *rep* genes in our dataset, and their most similar reference sequences, the *rep* sequences were 27–60% similar. The references with greatest similarity to the detected circoviruses were from viruses found in association with aquatic samples or invertebrates, including marine snails and hermit crabs, but also dragonflies (**Figure 4**).

FIGURE 3 | Circovirus-like contig ORFs Appeared in three different configurations (L) *Rep* types were present on three types of contig. (R) Predicted stem-loop structures associated with the RCR initiation nonanuclotide motif (9er) is highlighted.

FIGURE 4 | Alignment tree of *rep* ORFs in circovirus-like contigs.

DISCUSSION

We characterized the virome of both bleached and non-bleached individuals of the kelp *E. radiata*. Sequences similar to bacteriophages in the *Siphoviridae* were detected in greatest abundance overall, followed by *Myoviridae*, unclassified phages, *Inoviridae*, and *Podoviridae*. For most viral taxa there was little difference in the relative abundance in reads between the two kelp phenotypes. However, eukaryote circovirus-like sequences differed significantly in abundance between bleached and non-bleached kelp, with bleached viromes containing the vast majority (>99%) of circovirus-like reads. The circoviruses sequences from *E. radiata* included three phylogenetic groups, which all contained the characteristic *rep* gene of CRESS-DNA viruses, and were most similar to recently described circoviruses from pelagic water samples and marine invertebrates.

Initial alignment of raw DNA reads of kelp-associated viromes against a non-redundant protein database showed that most sequences were unknown, which is often the case in environmental metagenomes, but especially in marine virome data (Dinsdale et al., 2008a,b; Willner et al., 2009; Cassman et al., 2012; Brussaard et al., 2016). The most abundant contigs with similarity to known viruses were the bacteriophage *Caudovirales* (**Figure 2**). While the overall abundance of bacteriophage-like contigs did not differ, contigs similar to *Siphoviridae* were more abundant in viromes of non-bleached kelp, perhaps as a cause or consequence of the differences in the bacterial community previously seen between bleached and non-bleached *E. radiata* (Marzinelli et al., 2015). Large dsDNA viruses, such as *Phycodnaviridae* and *Mimiviridae*, formed the second largest group of virus-like contigs detected, and found with similar relative read numbers in metagenomes of non-bleached and bleached kelp. The majority of similarities to *Phycodnaviridae* were to EsV-1, which only produces virus particles in the reproductive structures of its host. EsV-1 may share a similar life history to *Phycodnaviridae* present in *E. radiata*, in which case the virus particles likely originated from the sori on the sampled kelp thallus. This group of viruses may be part of a persistent and possibly stable viral assemblage of the kelp holobiont. In contrast, significantly more circovirus-like sequences were detected in bleached kelp viromes than in non-bleached ones.

The circovirus genomes assembled here were of 2–5 kb in length. The small size of ssDNA virus genomes means that their genetic material is predisposed to be relatively scarce when compared to other viruses that may have up to 100-fold larger genomes (e.g., *Phycodnaviridae*). Whole genome amplification using multiple displacement amplification (MDA) of circular genomes has been used to sequence these viruses, even from very small amounts of DNA (Binga et al., 2008). MDA is known to create biases toward viruses with small genomes. Alternative methods, such as random priming-mediated sequence-independent single-primer amplification (RP-SISPA), could have been used, but this method can cause an overrepresentation in the datasets of viruses with large genomes (Adriaenssens

et al., 2017). This bias away from small genomes has been noted by other authors (Karlsson et al., 2013; Weynberg et al., 2014).

The genome structures of the CRESS-DNA virus sequences are similar to Type I and II ssDNA viruses, where the reading frames of the *rep* and capsid genes are positioned $3'–5'$ away from the stem-loop replication initiation structure. These viral types include *Circoviridae, Nanoviridae, Geminiviridae,* and other unclassified CRESS-DNA viruses from aquatic and marine metagenomes (Rosario et al., 2012b).

ssDNA (including CRESS-DNA) viruses have been characterized in medical and agricultural fields, due to their significance as human and agricultural pathogens (Roossinck, 2013). *Circoviridae, Anelloviridae, Genomoviridae,* and *Parvoviridae* are pathogens of humans and other vertebrates, such as pigs, poultry, and other domestic animals, but to date none have been identified in macroalgae. The circoviruses detected in the bleached kelp viromes were similar to a diverse subsection of marine viruses isolated from hermit crabs, sea anemones, but also had similarity to CRESS-DNA viruses detected in dragonflies (Rosario et al., 2012a). CRESS-DNA viruses are found in association with diverse habitats and host phyla (Rosario et al., 2012a,b; Eaglesham and Hewson, 2013; Fahsbender et al., 2015; Reavy et al., 2015), and circovirus-like proteins have been detected in a wide range of organisms, including mammals, birds, fish, cnidarians, as well as in a diverse range of aquatic and marine environments (Rosario et al., 2012b). Many circoviruses are important pathogens, or putative pathogens, and given their high relative abundance on the bleached phenotype it may be that they play a role in bleaching of *E. radiata*. Their increase may also be a secondary effect of bleaching, with the primary causative agent(s) being other microbes, or an interaction among multiple viruses, bacteria, fungi and others that are present in the kelp microbiome. Microbial agents can also interact with eukaryotic natural enemies, such as grazers to damage seaweeds (Campbell et al., 2014), which adds complexity when attempting to distinguish pathogens which cause disease from other secondary organisms (e.g., saprophytes such as fungi or bacteria) that arrive after disease onset to feed on compromised or decaying hosts (Burge et al., 2013; Egan and Gardiner, 2016). A more definitive resolution of the role of circoviruses in kelp bleaching thus awaits a more experimental approach, e.g., inoculation experiments, such as those conducted for *D. pulchra* and its bacterial pathogens (Campbell et al., 2015; Kumar et al., 2016). The circoviruses were the only eukaryotic DNA virus in the viromes that was overrepresented in the bleached compared with the non-bleached phenotypes, suggesting it may be associated with the bleached phenotype. However, ssDNA viruses have a ubiquitous presence in a wide range of eukaryotes and natural environments. The contigs we identified showed similarities to circoviruses found in hosts as broad as sea stars and crustaceans, raising the question of whether the circovirus was infecting the kelp host, or one of the herbivores associated with the kelp. Therefore, kelp could be infected with the circovirus, directly causing the phenotype, or the kelp could be acting as a vector to one of the herbivores or symbionts.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

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